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Increased expression of perforin, granzyme B, and C5b-9 in villitis of unknown etiology

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A B S T R A C T

Introduction: Villitis of unknown etiology (VUE) is associated with fetal growth restriction. However, the underlying mechanisms of villous injury in placentas with VUE are still largely unknown. We aimed to verify whether apoptosis-related factors are increased in VUE placentas. Furthermore, we determined apoptosis of villous cells.
Methods: Six placentas with VUE and 3 control placentas were stained using immunohistochemistry with antibodies for CD3, CD4, CD8, CD68, CD163, perforin, granzyme B, granzyme K, and C5b-9. TUNEL assay analysis was also performed with these placentas. The percentage of cells that stained positive, CD163/CD68 ratio, percentage of C5b-9 positive area, and apoptosis index were quantified and compared between the inflammatory lesions of the VUE placentas, non-VUE inflammatory lesions of the VUE placentas, and control placentas.
Results: The percentages of CD3, CD4, CD8 CD68, CD163, perforin, and granzyme B positive cells were significantly higher in the inflammatory lesions of the VUE placentas ($p < 0.05$). The intravillous CD163/CD68 ratio was higher in the inflammatory lesions compared with the non-inflammatory lesion of the VUE placentas ($p < 0.05$). The percentage of granzyme K-positive cells was not significantly different between the groups. C5b-9 deposition was higher in the inflammatory lesions of the VUE placentas ($p < 0.05$). TUNEL-positive cells were significantly higher in the inflammatory lesions of the VUE placentas ($p < 0.05$).
Discussion: To the best of our knowledge, this is the first report to assess villous injury, especially from a viewpoint of villous apoptosis in VUE placentas. An activated perforin/granzyme pathway and C5b-9 are suggested as possible mechanisms of apoptosis.

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1. Introduction

Villitis of unknown etiology (VUE) is an inflammatory condition of the placental parenchyma in which immunohistochemical and histochemical stains do not identify infectious agents such as *Toxoplasma gondii*, *Treponema*, cytomegalovirus, and the herpes simplex virus. The incidence of VUE is in the range of 5%–15% in third-trimester placentas [1]. VUE is associated with fetal growth restriction (FGR), intrauterine fetal death, unexplained premature delivery, preeclampsia, and perinatal asphyxia [2]; in particular, a number of studies have shown an association between VUE and FGR [3–6]. For example, in 44 placentas from FGR infants, Labarrer et al. reported that VUE was present in 86% of the placentas compared to 26% in those of appropriate-for-gestational-age infants [3]. However, the underlying mechanisms of villous injury in VUE placentas are still largely unknown.

Some studies suggest that VUE is the consequence of a maternal immune response against antigens present on the foreign fetal allograft; VUE is more frequent in multigravid mothers and associated with recurrent pregnancy complications [7,8]. Redline

Abbreviations: VUE, villitis of unknown etiology; FGR, fetal growth restriction; PBS, phosphate buffered saline.

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and Patterson studied the affected villi in VUE placentas from male infants. Using immunohistochemistry staining for CD3 and CD45 and in situ hybridization for X and Y chromosomes, they demonstrated the presence of maternal T-lymphocytes in the foci of the VUE placenta cells [9]. Although many other characteristics of the immune response have been clarified, the etiology of the eliciting antigen is unknown. The primary components of VUE are maternal T lymphocytes, predominantly CD8+ [10,11]. However, it has not been clarified how CD8+ T cells affect villous cells in VUE. The perforin/granzyme pathway is one of the cell death pathways activated by CD8+ T cells in which perforin and granzyme B are secreted by exocytosis from CD8+ T cells and together induce caspase dependent cell death of the target cells [12]. Granzyme K is also expressed in CD8+ T cells and induces caspase independent death of the target cells [13,14].

Several studies have also shown that the complement membrane attack complex C5b-9 induces apoptosis of target cells in various organs such as the heart, kidney, and placenta [15–17]. C5b-9 comprises a molecular complex of complement proteins, including C5b, 6, 7, 8, and 9. C5b-9 is typically formed on the surface of target cells as a result of the activation of the alternative pathway and classical pathway of the complement system. Excess binding of C5b-9 to the surface of cells induces inflammation and pore formation in the surface membrane [18]. However, the contribution of C5b-9 to VUE in placentas is poorly understood.

We hypothesized that villous apoptosis is induced by maternal inflammatory cells and complement activation in VUE. Hence, we sought to determine whether perforin and granzyme expressions and C5b-9 deposition is increased in VUE placentas. In addition, we evaluated the apoptosis of villous cells in VUE placentas.

2. Methods

2.1. Materials

Placental tissue samples used in this study were collected in the National Center for Child Health and Development between May 2007 and March 2013. Six placentas with VUE were retrospectively collected, and 3 gestational age-matched placentas without VUE were randomly chosen as control placentas; all placentas were collected with informed consent. The diagnosis of VUE was made when the mother, placenta, and infant did not show clinical, serological, or histologic evidence of infection known to be associated with chronic villitis. The clinical details of the cases are summarized in Table 1.

2.2. Histochemistry

More than 4 blocks were taken from each placenta. For each case, 3–4-μm thick sections of formalin-fixed and paraffin-embedded placental tissue were applied to poly-L-lysine glass slides. The sections were stained with hematoxylin and eosin and periodic acid-methenamine-silver stain. All of the sections were evaluated in a blinded manner by two pathologists (KM, AN).

2.3. Immunohistochemistry

The sections of tissue from single blocks in each of the 6 placentas with VUE and 3 control placentas were evaluated with immunohistochemistry using a panel of antibodies: murine monoclonal anti-CD3 (PS1; Nichirei, Tokyo, Japan; prediluted), murine monoclonal anti-CD4 (4B12; Nichirei, Tokyo, Japan; prediluted), murine monoclonal anti-CD8 (C8/144B; Nichirei, Tokyo, Japan; prediluted), murine monoclonal anti-CD20 (L26; Dako, Carpinteria, CA, USA; 1:200), murine monoclonal anti-CD56 (1B6; Leica, Wetzlar, Germany; 1:50), murine monoclonal anti-CD68 (PG-M1; Dako, Carpinteria, CA, USA; 1:20), murine monoclonal anti-CD163 (10D6; Novocastra, Newcastle, UK; 1:400), murine monoclonal anti-perforin (5B10; Novocastra, Newcastle, UK; 1:20), murine monoclonal anti-granzyme B (Grb-7; Dako, Carpinteria, CA, USA; 1:200), rabbit polyclonal anti-granzyme K (ab69884; Abcam, Cambridge, UK, 1:50), and murine monoclonal antiSC5b-9 (Quidel, San Diego, USA; 1:500). Sections stained by immunohistochemistry were studied at 400× magnification. Inflammatory lesions and non-inflammatory lesions of VUE were histologically diagnosed and confirmed by infiltration of CD3+ cells. The numbers of intravillous cells stained for CD3, CD4, CD8, CD20, CD56, CD68, CD163, perforin, granzyme B, and granzyme K were calculated in 4 inflammatory lesions and 4 non-inflammatory lesions. The numbers of CD68 and CD163 positive cells in the intervillous area, including the perivillous area, were also calculated in the same 4 inflammatory lesions and 4 non-inflammatory lesions. The percentage of cells within the villous core that stained positive was calculated. The intravillous and intervillous CD163/CD68 ratios were calculated to define the amount of CD163+ cells within the macrophage population. The CD163/CD68 ratio in each section was calculated as the number of CD163+ cells divided by the number of CD68+ cells. ImageJ 1.48 software was used to quantify the area positive for C5b-9 within the villous core as described previously [19].

2.4. TUNEL assay analysis

We quantified the apoptotic index using a TUNEL assay in the placental tissue from the same 6 VUE placentas and 3 control placentas according to the manufacturer's protocol (In Situ Cell Death Detection Kit; POD, Roche Diagnostic GmbH, Mannheim, Germany). After deparaffinization, the slides with the placental tissue were incubated in proteinase K working solution (2 μg/mL in 10 mmol/L Tris/HCL) at 37 °C for 20 min. The slides were then washed in phosphate buffered saline (PBS) prior to a 10-min incubation in 3% H_2O_2 in methanol. The slides were washed in PBS again and then incubated in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The slides were washed in PBS again, overlaid with 50 μL TUNEL reaction mixture, and incubated for 60 min at 37 °C. Thereafter, the sections were overlaid with 50 μL converter-peroxidase. All of the slides were stained with diaminobenzidine coupling and Mayer's Hematoxylin solution. Sections stained by the TUNEL assay were studied at 400× magnification. To assess apoptosis, nuclear staining of cells was examined, and the number of TUNEL-positive cells/field was counted to represent the apoptotic index [20,21].

2.5. Statistical analysis

Data were analyzed using the Mann–Whitney U and Kruskal–Wallis tests for non-parametric data using SPSS version 22.0 (IBM Corp., Armonk, NY, USA); a p-value <0.05 was considered significant.

3. Results

3.1. Cell analysis of VUE inflammatory lesions

The villous stroma of the villi was infiltrated by inflammatory cells in all of the inflammatory lesions of the VUE placentas (Fig. 1A). Syncytiotrophoblast detachment and basal membrane damage were also detected in the inflammatory lesions (Fig. 1A and B). The percentages of CD3, CD4, CD8, and CD68 positive cells were significantly higher in the inflammatory lesions of the VUE placentas, compared with the control placentas and non-inflammatory lesions of the VUE placentas (Figs. 1 and 2,

Table 1
Clinical characteristics of the study population.

Case no	Diagnosis	Maternal age (years)	Gravidity	Parity	Gestational age (weeks)	Fetal weight (g)	Placental weight (g)	Fetal Apgar score (1 min/5 min)
1	VUE	37	0	0	31	1344	277	5/9
2	VUE	32	0	0	39	1920	186	6/9
3	VUE	37	1	1	37	2072	232	8/9
4	VUE	30	2	0	38	2156	262	8/9
5	VUE	30	1	1	36	2100	353	8/9
6	VUE	32	0	0	39	2291	344	8/9
7	Without VUE	41	0	0	40	3078	467	8/9
8	Without VUE	34	3	0	33	1978	261	8/9
9	Without VUE	39	1	0	37	2586	299	8/9

VUE, villitis of unknown etiology.

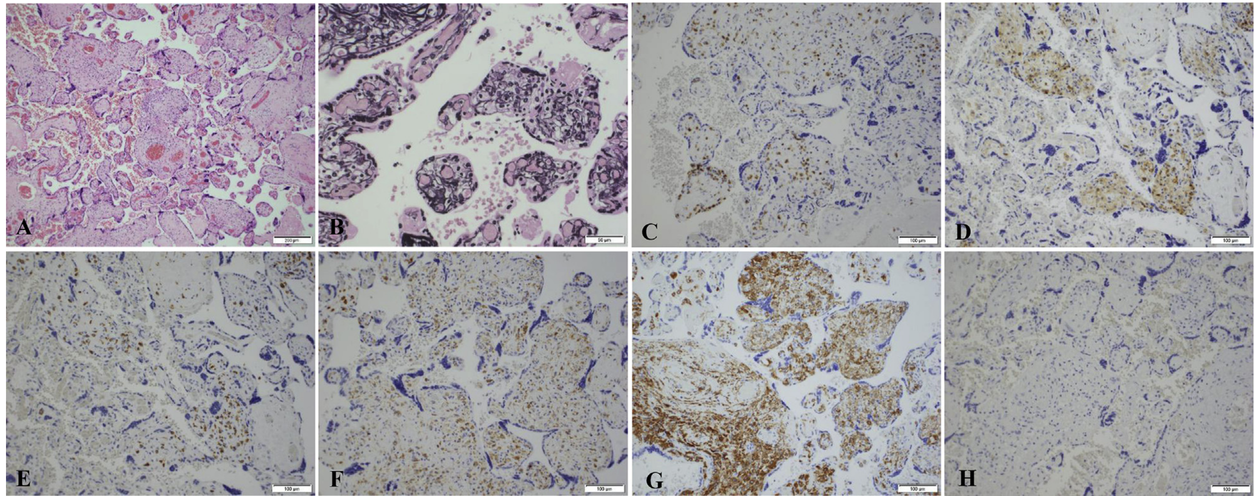

Fig. 1. Inflammatory lesion of a placenta with villitis of unknown etiology (VUE) (case 2). (A) At the VUE foci, mononuclear cells gather primarily in the terminal villi (hematoxylin and eosin, 10× magnification); (B) The basement membrane of the terminal villi is destroyed, and fibrinoid necrosis is visible in the perivillous lesion (periodic acid-methenamine-silver stain, 40× magnification); (C) CD3+ T lymphocytes aggregate at the perivillous and interstitium of the terminal villi (20× magnification); (D) CD4+ T lymphocytes are visible in the VUE foci, and the macrophages are also stained for CD4 (20× magnification); (E) CD8+ cytotoxic T lymphocytes aggregate in the VUE terminal villi (20× magnification); (F, G) Macrophages aggregate in the interstitium of the affected terminal villi (F: CD68, 20× magnification), (G: CD163, 20× magnification); (H) CD56 positive cells are not detected in the villitis (20× magnification).

$p < 0.05$). Staining for the M2 macrophage marker CD163 was significantly higher in the inflammatory lesions of the VUE placentas (Figs. 1G and 2E, $p < 0.05$). The intravillous CD163/CD68 ratio was significantly lower in the non-inflammatory lesions of the VUE placentas, compared with the control placentas (Fig. 3, $p < 0.05$). The intravillous CD163/CD68 ratio was significantly higher in the inflammatory lesions of the VUE placentas, compared with the non-inflammatory lesions of the VUE placentas (Fig. 3, $p < 0.05$). The median intervillous CD163/CD68 ratio in the inflammatory lesions of the VUE placentas was 0.58 (range, 0–0.85). Because there were almost no CD163+ and CD68+ cells present in the non-inflammatory lesions of the VUE placentas or the control placentas,

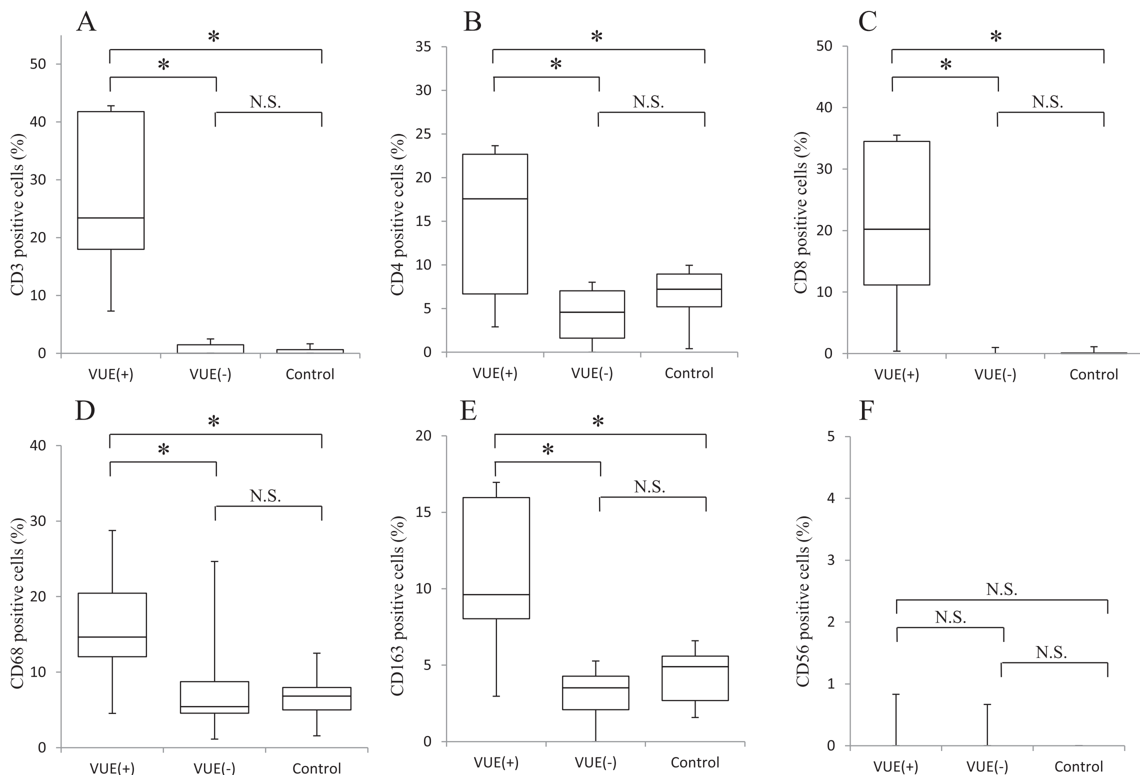

Fig. 2. Concentrations of (A) CD3, (B) CD4, (C) CD8, (D) CD68, (E) CD163, and (F) CD56 positive cells in inflammatory lesions of placentas with villitis of unknown etiology (VUE[+]), non-inflammatory lesions of VUE placentas (VUE[-]), and control placentas. Data are presented as median (line within the box), quartiles (25th and 75th percentiles are represented by the bottom and top of the box, respectively), and range (whiskers). Differences between the groups: *$p < 0.05$ and N.S., not significant.

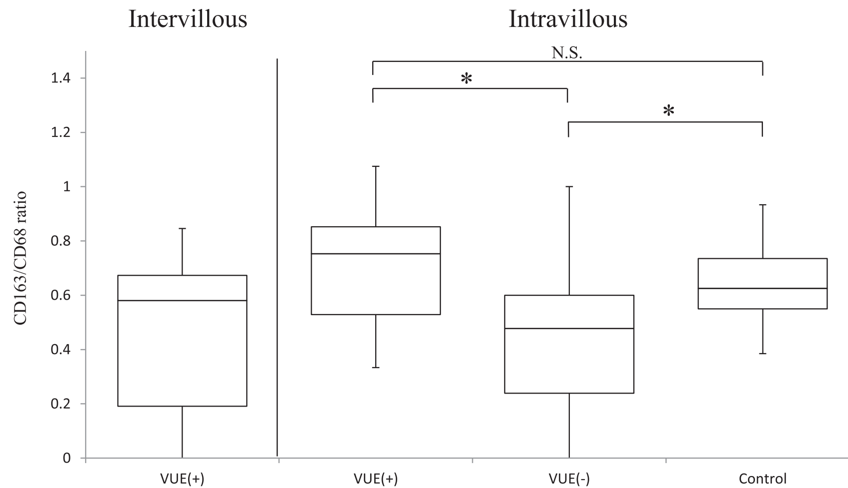

Fig. 3. Intervillous and intravillous CD163/CD68 ratio in inflammatory lesions of placentas with villitis of unknown etiology (VUE[+]), non-inflammatory lesions of VUE placentas (VUE[-]), and control placentas. Because there were almost no CD163+ and CD68+ cells in the non-inflammatory lesions of the VUE placentas and control placentas, the intervillous CD163/CD68 ratios were not calculated. Data are presented as median (line within the box), quartiles (25th and 75th percentiles are represented by the bottom and top of the box, respectively), and range (whiskers). Differences between the groups: *$p < 0.05$ and N.S., not significant.

the intervillous CD163/CD68 ratios were not calculated. Almost no CD56+ lymphocytes were identified in all of the placentas (Figs. 1H and 2F).

3.2. Perforin/granzyme pathway

Because the percentage of CD8+ lymphocytes was higher in the inflammatory lesions of the VUE placentas, we examined perforin, granzyme B, and granzyme K expression by immunohistochemistry. The percentages of perforin and granzyme B positive cells were significantly higher in the inflammatory lesions of the VUE placentas, compared with the control placentas and non-inflammatory lesions of the VUE placentas (Figs. 4 and 5A and B, $p < 0.05$). The percentage of granzyme K positive cells (Figs. 4 and 5C) was not significantly different between the groups.

3.3. C5b-9

The assessment of C5b-9 on villi as a potential factor for apoptosis identified slight C5b-9 deposition at the vascular smooth

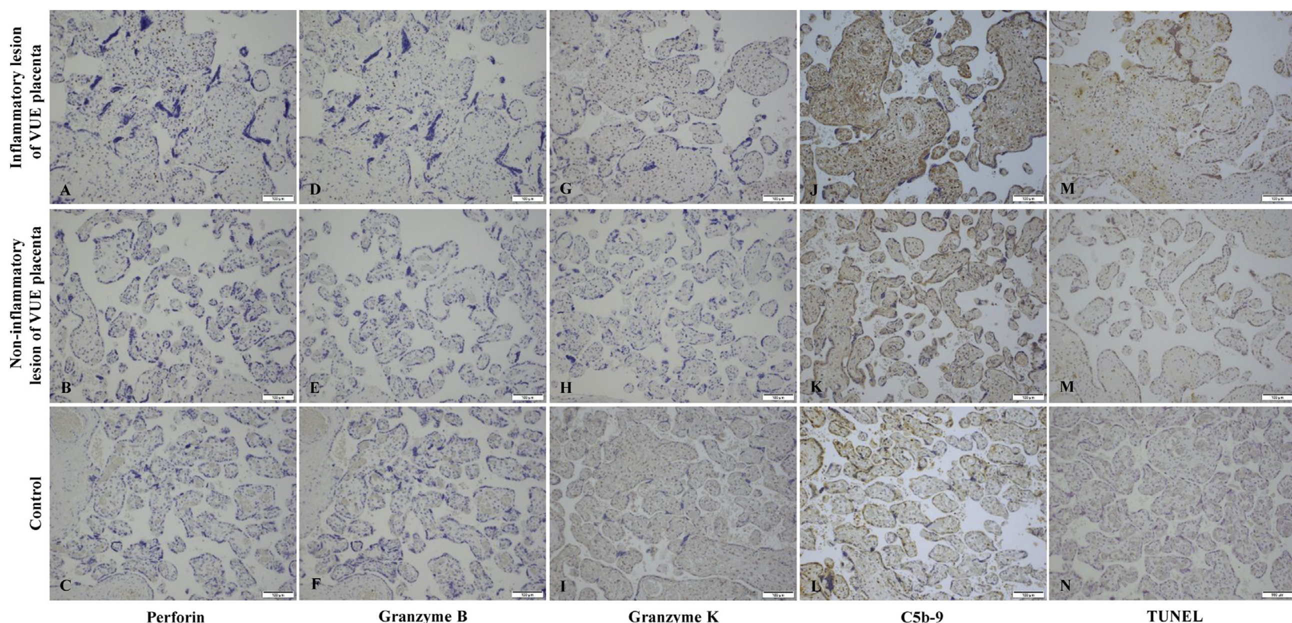

Fig. 4. Comparative analysis for the apoptotic pathway in an inflammatory lesion and non-inflammatory lesion of a placenta with villitis of unknown etiology (VUE) (case 2) and a control placenta (case 9). (A–C) Perforin positive cells are numerous in the inflammatory lesion of the VUE placenta; however, there are no perforin positive cells in the non-inflammatory lesion of the VUE placenta or control placenta; (D–F) Granzyme B findings are similar to those of perforin; (G–I) The presence of granzyme K is similar among the groups; (J–L) The C5b-9 positive area is significantly wider in the inflammatory lesion of the VUE placenta than in the non-inflammatory lesion of the VUE placenta and control placenta; (M–N) TUNEL positive signals are visible in the inflammatory lesion of the VUE placenta but not in the non-inflammatory lesion of the VUE placenta or the control placenta.

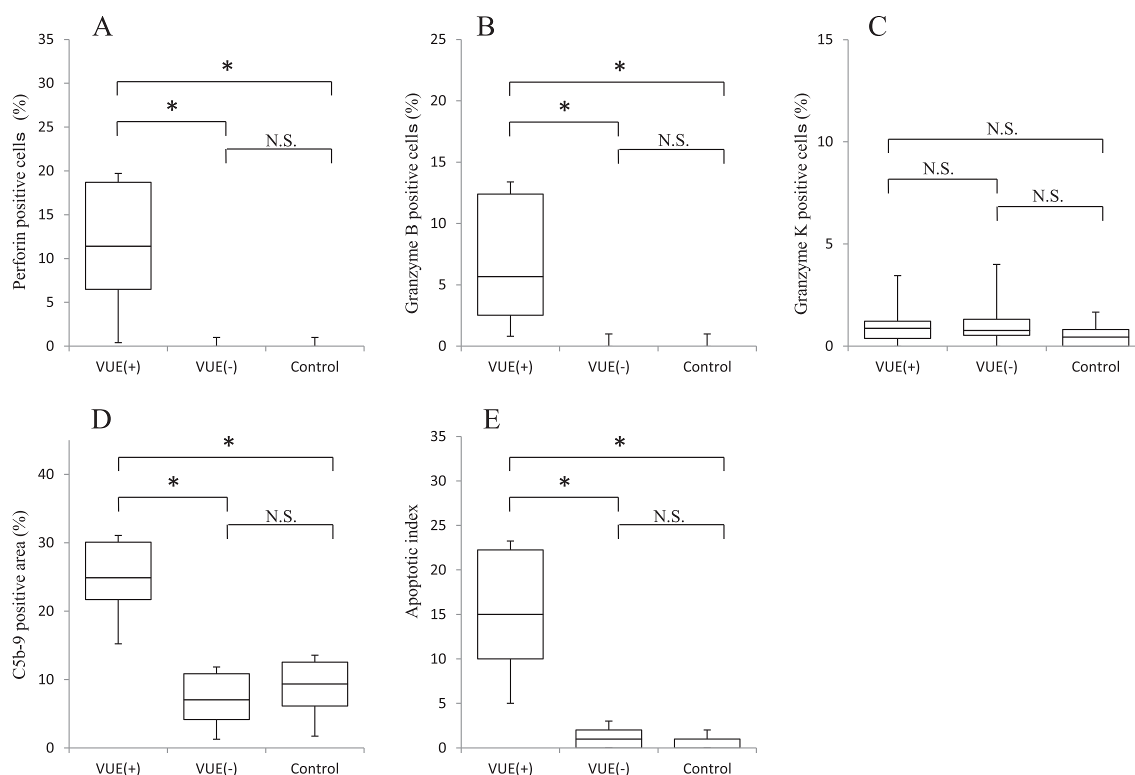

Fig. 5. Quantitative analysis of apoptosis-related factors. (A, B, C) Percentages of perforin, granzyme B, and granzyme K positive cells in inflammatory lesions of placentas with villitis of unknown etiology (VUE[+]), non-inflammatory lesions of VUE placentas (VUE[-]), and control placentas; (D) Percentage of the area positive for C5b-9; (E) Apoptosis index, calculated as the number of TUNEL-positive cells/field. Data are presented as median (line within the box), quartiles (25th and 75th percentiles are represented by the bottom and top of the box, respectively), and range (whiskers). Differences between the groups: *p < 0.05 and N.S., not significant.

muscle, trophoblasts, and fibrins around the villi in all of the placentas. In addition, high C5b-9 deposition was frequently found in the stromal areas of the inflammatory lesions of the VUE placentas. The percentage of the area that was C5b-9 positive was significantly higher in the inflammatory lesions of the VUE placentas, compared with the control placentas and non-inflammatory lesions of the VUE placentas (Figs. 4 and 5D, $p < 0.05$).

3.4. TUNEL analysis

Cytotrophoblasts, villous stromal cells, and endothelial cells yielded positive signals in the TUNEL analysis in all of the inflammatory lesions of the VUE placentas. There were few TUNEL-positive cells in the non-inflammatory lesions of the VUE placentas and control placentas, whereas TUNEL-positive cells were significantly higher in the inflammatory lesions of the VUE placentas (Figs. 4 and 5E, $p < 0.05$).

4. Discussion

Several studies have shown VUE to be associated with FGR [3–5], and all 6 VUE cases in the present study had FGR, with placental weights that were less than the normal range [22]. Villous injury resulting from VUE is suspected to influence fetal and placental growth. Because the underlying mechanisms of villous injury in VUE placentas are poorly understood, we investigated these mechanisms, particularly as they relate to villous apoptosis.

We found significantly higher expressions of perforin and granzyme B in the inflammatory lesions of the VUE placentas. Perforin is a cytolytic protein found in the granules of cytotoxic T lymphocytes that secrete granzymes into the cytosol of the target cell [23]. Several studies have shown that granzyme B secretion by cytotoxic T lymphocytes induces apoptosis of target cells [24,25]. In this study, granzyme B, but not granzyme K, was significantly higher in the inflammatory lesions of the VUE placentas, suggesting that apoptosis of intravillous cells was induced by granzyme B. Granzyme B is expressed primarily by cytotoxic CD8+ T cells [13,24]. However, several studies have shown that natural killer cells and cytotoxic CD4+ T cells also express granzyme B and induce apoptosis of the target cells [13,26,27]. We found significantly increased percentages of CD4+ T cells and CD8+ T cells, with almost no CD56+ cells in the inflammatory lesions of the VUE placentas. The high percentage of CD4+ cells might represent an increase in macrophages, because some CD68 positive macrophages are also positive for CD4 [28]. However, it is possible that not only cytotoxic CD8+ T cells but also cytotoxic CD4+ T cells induce apoptosis of the target cells in VUE placentas through the perforin/granzyme pathway.

C5b-9 deposition was also significantly higher in the inflammatory lesions of the VUE placentas. C5b-9 is the terminal complex of complement activation, and C5b-9 binding reportedly increases apoptosis in trophoblasts [17]. Previous studies have also shown that C4d deposition is higher in VUE placentas [29,30]. These findings suggest that dysregulated complement activation and C5b-9 deposition may be a mechanism for apoptosis of villous cells in VUE.

Similar to the study by Kim and Myerson, our study also showed that the number of macrophages is higher in inflammatory lesions of VUE placentas [11,31]. Of the macrophages, M1 macrophages are proinflammatory cells that produce proinflammatory cytokines and promote T helper 1 cell responses [32]. Conversely, M2 macrophages are immunosuppressive cells that are characterized by

their high production of anti-inflammatory cytokines such as IL-10 and high phagocytic activities of apoptotic cells [32]. The anti-inflammatory, tissue remodeling, and wound-repairing roles of M2 macrophages have been demonstrated previously in various tissues, including the central nervous system, heart, and lung [33–35]. Using immunohistochemistry staining of CD68, a general macrophage marker, and CD163, a specific M2 macrophage marker, we evaluated the ratio of M2 macrophages using CD163/CD68 and observed significantly greater staining of M2 macrophages in the inflammatory lesions of the VUE placentas. Using conjoint immunohistochemistry-in situ hybridization, Myerson et al. demonstrated that, in inflammatory lesions of VUE placentas, 89.5% of intravillous CD68+ macrophages are of fetal origin and 10.5% are of maternal origin, while intervillous CD68+ macrophages are universally of maternal origin [31]. Several reports have shown that decidual macrophages are mainly M2 macrophages; therefore, it is possible that the intravillous M2 ratio in the inflammatory lesions of the VUE placentas was increased by the invasion of maternal M2 macrophages [36,37]. However, the intervillous M2 ratio in the inflammatory lesions in the present study was not high (median, 0.58). Kim et al. showed that many macrophages in inflammatory lesions of VUE placentas are Ki67+ hyperplastic Hofbauer cells [11]. M2 macrophages might proliferate in the inflammatory lesions of VUE placentas. Although we could not specify the origin of the M2 macrophages in the present study, it is possible that fetal M2 macrophages act as immunosuppressors and phagocyte apoptotic cells in VUE placentas.

The intravillous M2 ratio in the non-inflammatory lesions of the VUE placentas was significantly lower than that in the control placentas, but the numbers of CD68+ cells and CD163+ cells were not significantly different. It is possible that the low intravillous M2 ratio of VUE placentas provided insufficient protection against the invasion of maternal inflammatory cells and tissue destruction. The M2 ratio of macrophages in the decidua reportedly contributes to the pathogenesis of preeclampsia [36]. We speculate that the low intravillous M2 ratio might contribute to the pathogenesis of VUE. However, additional studies are needed to determine why non-inflammatory lesions of VUE placentas are not affected by the lymphocytic infiltrate.

To the best of our knowledge, this is the first report to assess villous injury, especially from the viewpoint of villous apoptosis in VUE placentas. Activation of the perforin/granzyme pathway and C5b-9 are suggested as potential mechanisms of apoptosis.

Conflict of interest

None.

Acknowledgments

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